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Citation	Neuroscience Research, 56(3): 279-285
Issue date	2006-11
Type	Journal Article
URL	http://hdl.handle.net/2298/10010
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Receiving Editor: Dr. Seiji Ozawa

Contribution of endogenous glycine site NMDA agonists to excitotoxic retinal damage *in vivo*

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Number of Pages, 21; Number of Figures, 5; Number of Tables, 0

Abstract

N-methyl-D-aspartate (NMDA) receptors, which play an important role in neuronal excitotoxicity, require not only agonists at the glutamate-binding site but also co-agonists at the glycine site for their activation. Here we examined the role of endogenous agonists at the glycine site of NMDA receptors in excitotoxic retinal damage *in vivo*. To quantify the number of surviving retinal ganglion cells (RGCs), we injected a retrograde tracer, fluoro-gold, into the superior colliculus bilaterally and subsequently counted RGCs on whole-mounted retinas. Co-injection of 5'7-dichlorokynurenic acid (300 nmol), a competitive antagonist at the glycine site of NMDA receptors, rescued RGCs from damage induced by 200 nmol NMDA. On the other hand, RGC death induced by 20 nmol NMDA was enhanced by addition of glycine (10 nmol), D-serine (10 nmol) or a competitive glycine transporter-1 inhibitor, sarcosine (0.3 or 3 nmol). Moreover, application of D-serine-degrading enzyme, D-amino acid oxidase (30 mU), partially suppressed RGC death induced by 20 nmol NMDA. These results suggest that the severity of excitotoxic retinal damage *in vivo* depends on the levels of both glycine and D-serine.

Keywords: Apoptosis; Excitotoxicity; Ganglion cell; Glutamate; Neuronal death; Retina

1. Introduction

Glutamate is the major excitatory neurotransmitter in the retina and elsewhere in the central nervous system (CNS) (Kemp et al., 2002; Yang, 2004). Retinal bipolar cells send excitatory glutamatergic inputs to retinal ganglion cells (RGCs), which in turn transmit visual information to the brain via their axons that comprise the optic nerve. RGCs express distinct subclasses of glutamate receptors such as ionotropic *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors as well as metabotropic glutamate receptors (Yang, 2004). On the other hand, many lines of evidence suggest that pathological conditions in the retina including glaucoma (Nucci et al, 2005; Guo et al., 2006), ischemia (Adachi et al., 1998; Osborne et al., 1999) and optic neuropathy (Levkovitch-Verbin et al., 2003) are associated with dysregulation of glutamatergic system. Particularly, NMDA subtype of glutamate receptors is thought to play a principal role in neuronal degeneration in the retina, owing to its high Ca^{2+} permeability (Adachi et al., 1998; Lam et al., 1999). In an experimental rat glaucoma model, expression levels of glutamate transporters are decreased, which may result in elevation of extracellular glutamate concentration and over-activation of glutamate receptors (Martin et al. 2002; but see Hartwick et al., 2005).

Activation of NMDA receptor channels is well known to require a co-agonist at the glycine site, in addition to glutamate (Schell, 2004). At present, potential candidates for endogenous glycine site agonists in the retina are glycine and D-serine. Glycine is used as an inhibitory neurotransmitter by subpopulation of amacrine cells in the retina (Menger et al., 1998), but might also be supplied to the retinal tissue via peripheral circulation (Pow, 1998). More recently, the presence of another glycine site agonist D-serine as well as its synthesizing enzyme serine racemase has been proven in retinal astrocytes and Müller cells (Stevens et al., 2003). Several reports have clearly demonstrated that glycine and/or D-serine contribute to physiological activation of NMDA receptors in RGCs (Lukasiewicz and Roeder, 1995;

Stevens et al., 2003).

On the basis of their critical requirement for NMDA receptor activation, endogenous glycine site agonists may also play an important role under retinal pathological conditions involving excitotoxic events. In the brain, several studies demonstrated remarkable protective effects of glycine site antagonists against NMDA receptor-mediated neuronal injury (Foster et al., 1990; Patel et al., 1990). Recently, we and others have reported that endogenous D-serine contributes to NMDA-induced neuronal damage in cerebrcortical slices (Katsuki et al., 2004) and cultured hippocampal slices (Shleper et al., 2005). Here, we addressed whether endogenous glycine, D-serine, or both, regulate excitotoxic damage of RGCs *in vivo*, using a retinal degeneration model produced by intravitreal injection of NMDA.

2. Materials and methods

2.1. Drugs

We dissolved 5,7-dichlorokynurenic acid (DCKA; Tocris Cookson Inc., Bristol, UK) in 50% sodium hydroxide for intravitreal injection. The following drugs were dissolved in 0.1 M sterile phosphate buffer (pH 7.4) for intravitreal injection; NMDA (Sigma-Aldrich, St. Louis, MO), MK-801 (Sigma-Aldrich), glycine (Nacalai Tesque, Kyoto, Japan), D-serine (Nacalai Tesque), sarcosine (Nacalai Tesque) and D-amino acid oxidase (DAAOX; MP Biomedicals, Irvine, CA).

2.2. Intravitreal injection of drugs

Experiments were performed on male Sprague-Dawley rats (7 week old; Nihon SLC, Shizuoka, Japan). Animals were housed at 21 - 23 °C on a 12-h light-dark cycle (lights on at

8:00 AM). Food and water were freely available. All animal procedures were approved by our institutional animal experimentation committee and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Each rat was anesthetized with an intraperitoneal injection of sodium pentobarbital (50 - 70 mg/kg). We carried out intravitreal injection using a 33-gauge needle connected to a Teflon tube with a 25-μL Hamilton syringe after dilation of the rat pupil with 1% atropine sulfate. The tip of the needle was inserted through the dorsal limbus of the eye. We injected 5 μL of a drug solution slowly over a period of 1 min (Adachi et al., 1998; Takahata et al., 2003).

2.3. Analysis for retinal ganglion cell loss

We labeled surviving RGCs in a retrograde manner, essentially according to the methods described previously (Takahata et al., 2003). Three days after intravitreal injection of drugs, each rat was anesthetized by an intraperitoneal injection of sodium pentobarbital (50 - 70 mg/kg), and the head was placed firmly in a stereotaxic apparatus. We injected 2.5 μL of a solution of 4% fluoro-gold (FG; Fluorochrome, LLC, Colorado, USA) bilaterally into the superior colliculus (–6.3 mm posterior to bregma, ±1.5 mm lateral to the midline, –3.6 mm from the surface of the skull).

Four days after FG injection (e.g., seven days after intravitreal injection of drugs), the rat was euthanized, and both eyes were enucleated. The eyes were fixed for 45 min in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The retinas were dissected as flattened whole mounts with four radial cuts, mounted with vitreal side up on slides and covered with Gel/Mount (Biomeda Corp., Foster City, CA, USA).

To count FG-labeled RGCs, we randomly selected four fields of 230×310 μm^2 from the central area (approximately 1 mm from the optic disc) and the peripheral area (more than 3 mm from the optic disc) for each retina. FG-labeled RGCs were counted with the aid of

image analysis software (Scion Image). For each animal, the mean value for the right eye was expressed as a ratio (%) to that for the intact left eye. Data are expressed as mean ± SEM. Statistical analyses were performed with one-way analysis of variance followed by Student-Newman-Keuls' test, and differences were considered significant at $P < 0.05$.

Although sampling methods for cell counting, using retinal cross sections or a fractional area of retinal flat mounts like in the present study, have been used widely (Huang et al., 2005; Manabe et al., 2005), several problems associated with these methods have been claimed by Danias et al. (2002, 2006). The degree of RGC damage is highly variable in the case of the vein cauterization model of glaucoma in the rat (Danias et al., 2006), which necessitates a large scale of cell counting to draw statistically significant results. On the other hand, we observed during inspection of retinal whole mounts that NMDA cytotoxicity and the effects of drugs described below were consistent between different animals, and radial asymmetry of drug effects was not apparent within a given retina. Therefore, the method of cell counting should not produce a bias that may affect the conclusion of the present study.

2.4. Histological analysis

In several sets of experiments, we examined histological alterations in retinal tissues by conventional hematoxylin/eosin staining (Takahata et al., 2003). Seven days after intravitreal injection of drugs, each rat was euthanized and both eyes were enucleated. We fixed the eyes in phosphate-buffered 4% formalin and 1% glutaraldehyde aqueous solution (pH 7.4), and then in phosphate-buffered 10% formalin solution (pH 7.4). After fixation, the eyes were embedded in paraffin and cut into 5-μm-thick sections through the optic disc of each eye. The sections were then stained with hematoxylin and eosin (HE). We took photomicrographs of each section within 1 mm of the optic disk.

Occurrence of DNA fragmentation was examined by terminal deoxynucleotidyl

transferase-mediated dUTP nick end-labeling (TUNEL). At 18 h after intravitreal drug injection, each rat was euthanized and both eyes were enucleated. After fixation, the eyes were embedded in paraffin and cut into horizontal 5-μm-thick sections through the optic disc of each eye. We applied *In situ* Apoptosis Detection Kit (Takara Bio Inc., Shiga, Japan) to paraffin sections, according to the manufacturer's protocol with slight modification. Briefly, FITC-dUTP was catalytically added by TdT to the 3'-OH ends of double- or single-stranded DNA. The sections were then incubated with Anti-FITC HRP conjugate at 37°C overnight. Following these processes, the sections were incubated with anti-rabbit IgG (Vector, Burlingame, CA) and then with ABC solution (ABC Elite Kit; Vector) at room temperature, respectively for 2 h. We visualized the labeled product with diaminobenzidine that yielded brown granules localized to apoptotic cells. Finally, we counterstained the sections with 1% methyl green. Omission of TdT gave completely negative results.

3. Results

3.1. Effect of glycine site blockade on NMDA-induced retinal damage

As previously described (Takahata et al., 2003), intravitreal injection of 200 nmol NMDA caused a prominent decrease in the number of FG-labeled RGCs assessed at 7 days after injection (Fig. 1A). The degree of the decrease was comparable between the central area and the peripheral area. To ascertain whether the presence of endogenous glycine site agonists is critical for NMDA cytotoxicity, we examined the effect of DCKA (30 - 300 nmol) on retinal damage induced by 200 nmol NMDA. DCKA is a potent antagonist acting at the glycine site of NMDA receptor complex (Baron et al., 1990). DCKA at 300 nmol almost completely abolished NMDA-induced loss of RGCs both at the central area and the peripheral area (Fig. 1A and B). The effect of lower doses of DCKA was modest, but a significant

effect of 30 nmol DCKA was observed at the peripheral area.

Consistent with results on whole-mounted retina, histological analysis of transverse retinal sections with HE staining revealed that 200 nmol NMDA caused cell loss in the ganglion cell layer and also a marked reduction in the thickness of the inner plexiform layer 7 days after injection (Fig. 2A). Co-injection of 300 nmol DCKA with NMDA completely rescued cells in the ganglion cell layer and preserved the thickness of the inner plexiform layer. TUNEL of transverse retinal sections revealed that positively-stained cells, which were not evident in vehicle-treated retinas, were distributed in the ganglion cell layer (GCL) at 18 h after intravitreal injection of 200 nmol NMDA (Fig. 2B). At this time point, cell loss in GCL and decrease in the thickness of the inner plexiform layer were not prominent, indicating that apoptotic processes precede NMDA-induced degeneration of RGCs. Co-injection of 300 nmol DCKA with NMDA eliminated the appearance of TUNEL-positive cells in GCL (Fig. 2B). We also noted appearance of many TUNEL-positive cells in the inner nuclear layer (INL), which was also eliminated by DCKA. This is consistent with the fact that amacrine cells located in this layer are vulnerable to NMDA cytotoxicity (Lam et al., 1999). The apparently large number of TUNEL-positive cells in INL is likely to reflect a much larger number of cells in this layer than in GCL, and the percentage of cell loss in INL seemed to be very low (Fig. 2A). We did not perform further examinations on INL, because dense package of cells in this layer hindered accurate assessment of the number of surviving cells.

3.2. Effects of glycine site agonists on NMDA-induced RGC death

Several lines of evidence suggest that the glycine site of NMDA receptors is not saturated in the CNS, including the retina (Chen et al., 2003; Hashimoto et al., 1992; Stevens et al., 2003). To address this possibility, we examined whether exogenous application of

glycine site agonists could influence the degree of NMDA cytotoxicity. As shown in Fig. 3A, co-injection of 10 nmol glycine significantly exacerbated RGC death induced by 20 nmol NMDA at the peripheral area. Glycine also tended to exacerbate RGC death at the central area, although the effect did not reach statistical significance. On the other hand, co-injection of 10 nmol D-serine significantly exacerbated RGC death by 20 nmol NMDA at both the central and the peripheral areas (Fig. 3B). However, neither glycine nor D-serine influenced excitotoxic consequences induced by 200 nmol NMDA. Neither glycine nor D-serine alone affected the viability of RGCs.

3.3. Effect of a glycine transporter-1 inhibitor on NMDA-induced RGC death

Na^+/Cl^--dependent, high-capacity glycine transporters (GLYTs) have been reported to regulate extracellular glycine concentrations efficiently in the forebrain (Chen et al., 2003). Two subtypes of GLYTs encoded by different genes, *GlyT1* and *GlyT2*, are differentially expressed in the CNS (Zafra et al., 1995). In the retina, distribution of GLYT1 matches that of glycine-immunoreactive amacrine cells, whereas GLYT2 is absent from the retina (Zafra et al., 1995; Menger et al., 1998). Thus, we examined the effect of sarcosine, a competitive inhibitor of GLYT1 (Guastella et al., 1992), on NMDA-induced RGC death. Co-injection of 0.3 or 3 nmol sarcosine and 20 nmol NMDA 1 day after injection of the same doses of sarcosine induced more severe RGC death than injection of 20 nmol NMDA alone 1 day after vehicle injection (Fig. 4). The exacerbating effect of sarcosine on RGC death was significant only at the central area of the retina, although tendency for exacerbation was observed also at the peripheral area.

3.4. Effect of D-serine-degrading enzyme on NMDA-induced RGC death

In the final set of experiments, we examined the effect of DAAOX on

NMDA-induced RGC death, to verify whether D-serine engages in excitotoxicity as an endogenous glycine site agonist in the retina. DAAOX is an enzyme that catalyzes oxidative deamination of D-amino acids (D'Aniello et al., 1993; Denu and Fitzpatrick, 1994). At physiological pH, this enzyme is highly selective for D-serine and does not act on other amino acids, including glycine. We intravitreally injected DAAOX and 20 nmol NMDA 1 day after injection of the same dose of DAAOX. The results presented in Fig. 5 show that 30 mU DAAOX significantly reduced RGC death induced by NMDA at the peripheral area of the retina. The protective effect of DAAOX was not prominent at the central area.

4. Discussion

In the present study, we aimed at uncovering the characteristics of retinal excitotoxicity by focusing on the glycine site of NMDA receptors. First of all, we examined the effect of DCKA, a potent competitive antagonist at the glycine site (Baron et al. 1990). DCKA at a sufficient dose applied with NMDA almost completely blocked loss of RGCs, thinning of the inner plexiform layer, and occurrence of DNA fragmentation revealed by TUNEL. These results clearly indicate that glycine site stimulation by endogenous ligands is obligatory for NMDA-induced retinal damage. The effects of DCKA observed in this study are not surprising, given the fact that a glycine site ligand is essential for activation of NMDA receptors (Schell, 2004). To our knowledge, however, protective effect of glycine site antagonists against NMDA-induced retinal damage *in vivo* has not been investigated directly. The only relevant report is that systemic administration of kynurenine, a precursor of a glycine site antagonist kynurenic acid, attenuates NMDA cytotoxicity in rat retina (Vorwerk et al., 1996).

The second question we addressed was whether endogenous glycine site ligands are

present extracellularly in the retina at levels sufficient for full activation of NMDA receptors. Results of co-injection experiments showed that D-serine and glycine exacerbated RGC death induced by 20 nmol NMDA. These results suggest that the glycine site of NMDA receptors on RGCs *in vivo* is not normally saturated, and that changes in the extracellular levels of glycine site ligands can influence the degree of excitotoxic damage. Unsaturated properties of the NMDA receptor glycine site have been suggested in isolated retinal tissues (Stevens et al., 2003), but endogenous ligands might have been partially wiped out by external perfusion in *in vitro* preparations.

D-Serine significantly exacerbated NMDA cytotoxicity both at the central area and at the peripheral area of the retina, whereas glycine showed a significant effect only at the peripheral area. This difference may be attributable to the fact that glycine is a weaker agonist than D-serine at the NMDA receptor glycine site (Matsui et al., 1995). Another interpretation is a potential difference in the distribution of uptake systems of glycine and D-serine. Different types of transporters participate in clearance of extracellular glycine and D-serine. That is, a glycine transporter GLYT1 expressed on retinal amacrine cells (Zafra et al., 1995; Menger et al., 1998) plays an important role in regulation of synaptic glycine concentration (Chen et al., 2003) and glycine content in retinal neurons (Pow, 1998). NMDA receptor-mediated transmission is augmented in hippocampal CA1 synapses in heterozygous GLYT1 knockout mice (Tsai et al., 2004; Gabernet et al., 2005), indicating that GLYT1 maintains extracellular glycine concentration below the levels saturating NMDA receptor glycine site. On the other hand, uptake of extracellular D-serine in the retina is probably accomplished by ASCT-like neutral amino acid transporters (O'Brien et al., 2005). Therefore, the effects of exogenously applied glycine site agonists may well be influenced by activities of these transporters. Unfortunately, however, there is no information available whether or not the expression levels or activities of these transport systems exhibit any

regional differences within the retinal tissues.

In contrast to that induced by 20 nmol NMDA, RGC death induced by a higher dose (200 nmol) of NMDA was not exacerbated by glycine and D-serine. Reasons for this insensitivity are unclear, but a possible explanation is that a subpopulation of RGCs is resistant to NMDA cytotoxicity (Dreyer et al., 1994). Thus, a high dose of NMDA is sufficient to damage almost all NMDA-sensitive RGCs even in the presence of only modest levels of endogenous glycine site agonists. In this case, supplementation of glycine site agonists may no longer increase the number of damaged RGCs, because the remaining population of RGCs is relatively NMDA-resistant.

Effects of sarcosine and DAAOX on NMDA-induced RGC death provide further evidence for important roles of endogenous glycine site agonists. Sarcosine, a competitive inhibitor of GLYT1, exacerbated RGC death induced by 20 nmol NMDA, indicating that regulation of extracellular glycine levels by GLYT1 limits over-activation of NMDA receptors leading to retinal damage. Interestingly, the effect of sarcosine was more prominent in the central area than in the peripheral area. Combined with differential effects of exogenous glycine on NMDA cytotoxicity between different areas (Fig. 3A), these results imply that glycine transport activity exerts more profound influences on regulation of extracellular glycine in the central area than in the peripheral area of the retina.

In contrast to the effect of sarcosine, the effect of DAAOX on NMDA-induced retinal damage was significant only in the peripheral area. These results offer an interesting possibility that different kinds of endogenous glycine site agonists play a dominant role in the central and the peripheral area of the retina: that is, glycine dominates in the central area whereas D-serine dominates in the peripheral area. Again, however, no information is available concerning regional distribution of D-serine and serine racemase, the D-serine synthesizing enzyme, within the retinal tissues. In any case, the present results demonstrated

for the first time that endogenous D-serine is at least in part involved in pathological processes mediated by NMDA receptor over-activation in the retina. To our knowledge, the present study is also the first one suggesting that D-serine plays an active role in excitotoxicity *in vivo*.

Glial cells are emerging as important participants in various neurodegenerative conditions including excitotoxic injury (Kim and de Vellis, 2005; Seifert et al., 2006). With regard to glycine site ligands, Müller cells and astrocytes in the retina are potential sources of D-serine (Stevens et al., 2003). Moreover, glial cell activation is associated with glaucoma (Wang et al., 2000), and activated microglia may serve as an additional source of D-serine (Wu and Berger, 2004). Thus, states of glial cells (quiescent or activated) may be taken into consideration to understand the roles of endogenous glycine site ligands under pathological conditions.

Overall, the levels of endogenous glycine site agonists influence the severity of NMDA receptor-mediated excitotoxic retinal damage *in vivo*. Both glycine and D-serine contribute to the excitotoxic processes in the retina, but their relative contribution may not be uniform throughout the retinal tissue. Further studies are required concerning distribution and activities of key molecules involved in the synthesis, release and uptake of glycine and D-serine within the retina. These kinds of information should provide useful insights into the regulatory mechanisms of excitotoxicity, which are relevant to the pathogenesis of various retinal neurodegenerative disorders. Finally, we must note that findings in the present study rely solely on morphological analysis. Whether manipulation of the extent of glycine site stimulation under pathological conditions leads to preservation of retinal functions, e.g., as reflected by electroretinograms, remains an important issue to be determined.

Acknowledgements

This study was supported by Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science and The Ministry of Education, Culture, Sports, Science and Technology, Japan, and also supported by a grant from Astellas Foundation for Research on Medicinal Sources.

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Figure legends

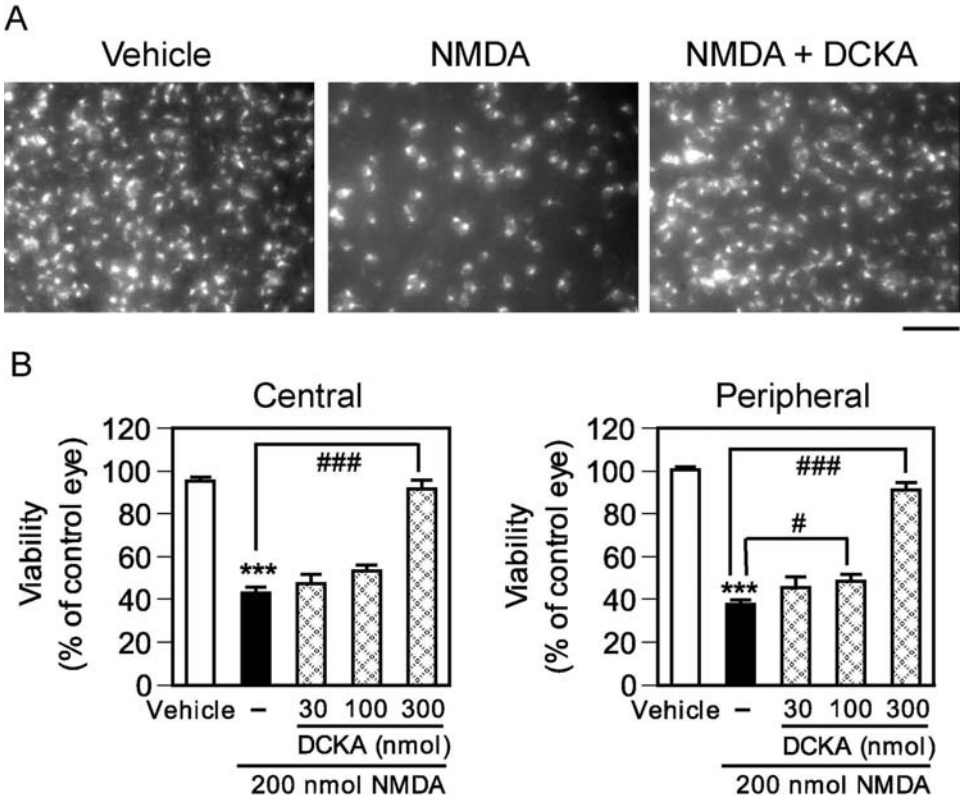
Fig. 1. Effect of a glycine site antagonist on NMDA-induced retinal damage. A, Representative photomicrographs of the central area of whole-mounted preparations of retinas that received intravitreal injection of vehicle, 200 nmol NMDA, or 200 nmol NMDA plus 10 nmol DCKA. Scale bar, 50 μm. B, Summary of the effect of DCKA on NMDA-induced loss of RGCs at the central and the peripheral areas 7 days after intravitreal injection. Means of the number of FG-labeled surviving RGCs for the right eye of each animal were normalized to those for the left (intact) eye and are shown as percentages. *** $P < 0.001$ vs. vehicle. # $P < 0.05$, ### $P < 0.001$. n = 9 - 11.

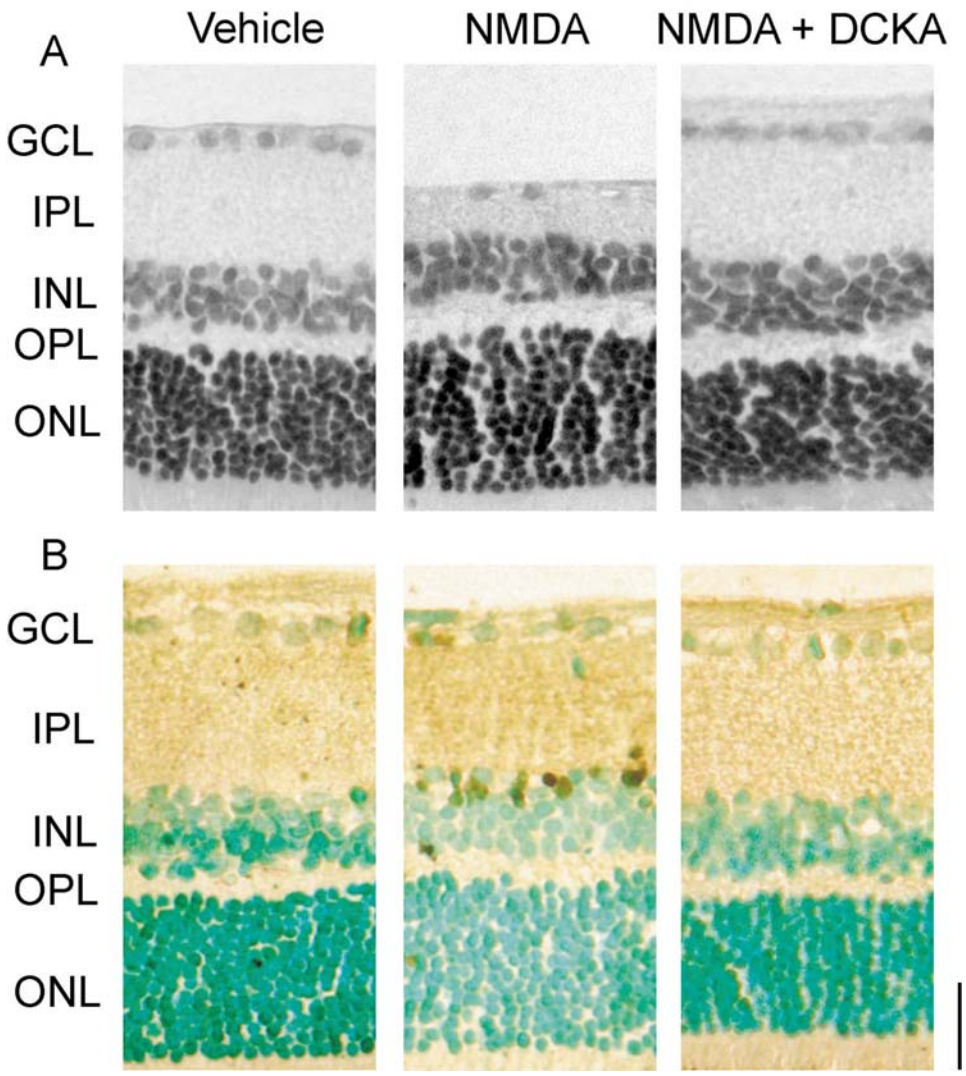
Fig. 2. Representative photomicrographs of hematoxylin-eosin (HE) -stained (A) and TUNEL-stained (B) retinal sections from eyes that received intravitreal injection of vehicle (left), 200 nmol NMDA (center) or 200 nmol NMDA plus 300 nmol DCKA (right). Eyes were enucleated at 18 h or 7 days after injection, and then TUNEL staining or HE staining was performed respectively. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar, 20 μm.

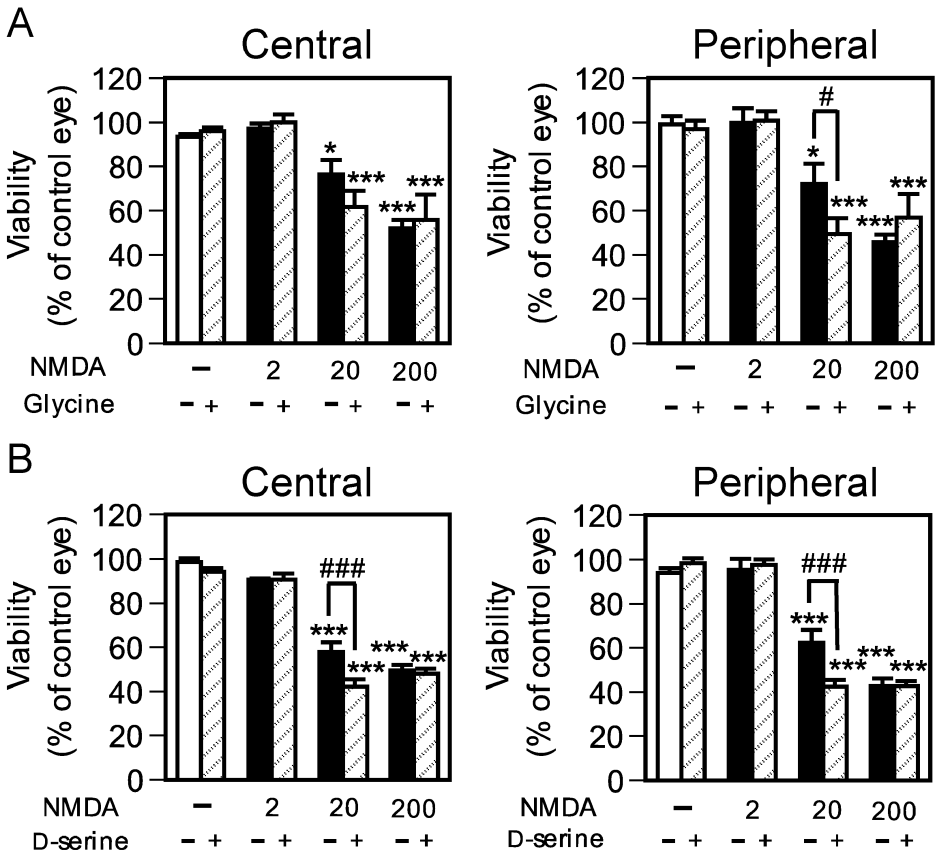
Fig. 3. Effects of glycine site agonists on NMDA-induced RGC death 7 days after intravitreal injection. Effect of glycine (10 nmol; A) and D-serine (10 nmol; B) at the central and the peripheral areas are shown. Glycine site agonists were simultaneously applied with NMDA at indicated doses (in nmol). Means of the number of FG-labeled surviving RGCs for the right eye of each animal were normalized to those for the left (intact) eye and are shown as percentages. * $P < 0.05$, *** $P < 0.001$ vs. vehicle. # $P < 0.05$, ### $P < 0.001$. n = 5 - 8.

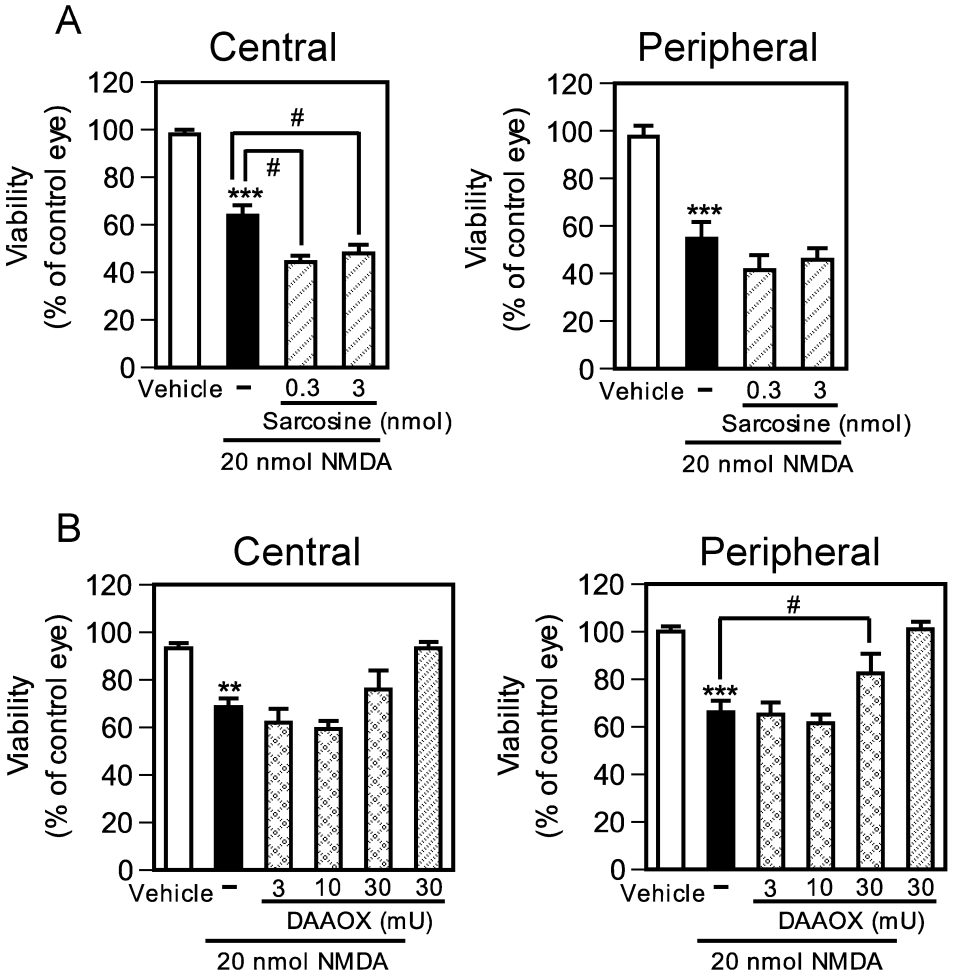
Fig. 4. Effect of a glycine transporter inhibitor on NMDA-induced RGC death 7 days after intravitreal injection. Sarcosine at indicated doses was injected intravitreally, and 24 h later, it was again injected simultaneously with 20 nmol NMDA. Means of the number of FG-labeled surviving RGCs for the right eye of each animal were normalized to those for the left (intact) eye and are shown as percentages. *** $P < 0.001$ vs. vehicle. # $P < 0.05$. n = 6 - 9.

Fig. 5. Effects of a D-serine degrading enzyme on NMDA-induced RGC death 7 days after intravitreal injection. DAAOX at indicated doses was injected intravitreally, and 24 h later, it was again injected simultaneously with 20 nmol NMDA. Means of the number of FG-labeled surviving RGCs for the right eye of each animal were normalized to those for the left (intact) eye and are shown as percentages. ** $P < 0.01$, *** $P < 0.001$ vs. vehicle. # $P < 0.05$. n = 4 - 8.


A
Vehicle
NMDA
NMDA + DCKA
B
Central
Peripheral
Viability
(% of control eye)
120
100
80
60
40
20
0
###
#
***
Vehicle
–
30
100
300
DCKA (nmol)
200 nmol NMDA


Vehicle
NMDA
NMDA + DCKA
A
GCL
IPL
INL
OPL
ONL
B
GCL
IPL
INL
OPL
ONL


A
Central
Peripheral
Viability
(% of control eye)
120
100
80
60
40
20
0
NMDA
Glycine
–
2
20
200
– +
*
***
#
B
###
D-serine


A
Central
Peripheral
Viability
(% of control eye)
120
100
80
60
40
20
0
#
#
***
***
Vehicle
–
0.3
3
Sarcosine (nmol)
20 nmol NMDA
B
Central
Peripheral
**
***
#
Vehicle
–
3
10
30
30
DAAOX (mU)
20 nmol NMDA